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DISTRIBUTION OF SEROTONIN-LIKE IMMUNOREACTIVITY IN THE CENTRAL NERVOUS SYSTEM OF THE PERIWINKLE, *LITTORINA LITTOREA* (GASTROPODA, PROSOBRANCHIA, MESOGASTROPODA)

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Abstract

The distribution of serotonin-like immunoreactivity was determined in whole-mounted ganglia of the central nervous system of the prosobranch snail, *Littorina littorea,* with the goal of establishing a basis for cellular comparisons between different gastropod nervous systems. While general similarities appear to exist with other species, the nervous system of *Littorina* appears to differ markedly from the nervous systems of the pulmonate and the opisthobranch gastropods with regard to its relatively large number of small, serotonergic cell bodies. Over 1500 cells in *Littorina* were tentatively identified as serotonergic. These cells and their processes were distributed through all the major ganglia, but the vast majority of immunoreactive cell bodies were found in the pedal ganglia. Most cells in the central nervous system were identified only as members of clusters, however, a few could be reliably identified as consistent individuals between preparations. Further histological and physiological studies are needed on prosobranch nervous systems before evolutionary relationships can be established at the cellular level with pulmonate or opisthobranch nervous systems.

Introduction

The gastropod nervous system has developed through complex evolutionary processes which are poorly understood. A major reason for this lack of understanding is that homologies between nerves, ganglionic lobes, and even whole ganglia, which could serve as the basis for such an understanding, are often not obvious because of changes rendered by cephalization, torsion, and detorsion (Bullock and Horridge, 1965). Recently, however, some insights have been gained from the finer resolution afforded by the study of single identified neurons. For example, the examination of cells which appear to be bilateral homologs, but which appear to lie within bilaterally asymmetrical ganglia, have provided new means for identifying parts of ganglia which may have fused through evolution (Hughes and Tauc, 1963; Hughes, 1967; Munoz *et al.,* 1983). Single cells which are putative homologs have also been identified between species. While the serotonergic metacerebral giant cell is the best studied example of such a neuron in gastropods (Senseman and Gelperin, 1974; Weiss and Kupfermann, 1976; McCrohan and Benjamin, 1980; Granzow and Rowell, 1981; Pentreath *et al.,* 1982; Longley and Longley, 1984; Croll, 1985), evidence suggesting other homologies has also appeared (Dorsett, 1974; Chase and Goodman, 1977; Dickinson, 1980a, b). Such findings may provide useful clues into certain phylogenetic relationships, but

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this body of literature is presently too sparce to serve as a solid basis for understanding broad evolutionary trends in the gastropod nervous system.

One direction in which to expand the present scope of the literature is toward a wider phyletic approach. Over the last two decades, there has been a tremendous expansion in our knowledge of the anatomy and physiology of the gastropod nervous system. To date, however, studies have been relatively restricted within the gastropods, with most examples having been drawn from only a small portion of the existing opisthobranch and the pulmonate genera. Large gaps, therefore, still exist in our knowledge. In fact, very little has changed in intervening years to invalidate the statement of Bullock and Horridge (1965) that only a few "sketchy results are about the only histological studies in the brain in the whole range of prosobranchs" (p. 1359).

As a first effort at building a broader comparative base, the present report demonstrates the location of cell bodies and their processes with serotonin-like immunoreactivity in the central nervous system of the periwinkle, *Littorina littorea.* Since the distribution of serotonergic cells is now established in a number of other gastropods, the purpose of this study is to establish a basis for comparison at the cellular level. Such a comparison has an added evolutionary importance since *Littorina,* a fairly typical mesogastropod prosobranch, is thought to represent the more ancestral form from which both the pulmonates and the opisthobranchs may be derived (Bullock and Horridge, 1965).

Since the periwinkle has not been used as a modern neurobiological preparation, aspects of its gross neuroanatomy are re-examined here and are compared with previous reports as a necessary background for the interpretation of the observed serotonin-like immunoreactivity patterns.

A preliminary report of certain aspects of this study has appeared elsewhere (Croll, 1985).

Materials and Methods

Adult *Littorina littorea* were collected locally and were maintained at ambient temperatures (3°C–13°C) in fresh, running seawater supplied by the Dalhousie Aquatron System.

Gross neuroanatomy

Five animals were sacrificed for an examination of the gross anatomy of the central nervous system. After dissection, the ganglia were placed on microscope slides according to their *in situ* orientation, with the exception of the pedal ganglia which were rotated 90° so that the posterior surface faced upward for flat mounting. The ganglia were then stained with methylene blue, dehydrated, cleared and mounted with Permount in preparation for camera lucida tracing.

Immunohistochemical procedures

These procedures were based largely on the indirect immunofluorescent techniques developed by Coons (1958) and subsequently applied to the detection of serotonin-like immunoreactivity by Steinbusch *et al.* (1978). Further refinements for use with whole-mounted invertebrate ganglia were added by Beltz and Kravitz (1983). In addition, the ganglia were treated with protease prior to fixation (see below) to allow for better penetration of antibodies.

For normal immunocytochemical staining, the central ganglia were dissected from approximately 50 snails. The ganglia were cleaned of loose, surrounding tissue

but no attempt was made to mechanically remove the inner connective tissue sheath. The ganglia were next immersed in 0.5% protease (Type IV, Sigma Chemical) in phosphate buffered saline (PBS:13.4 g $Na_2HPO_4 \cdot 7H_2O$ and 8.0 g NaCl diluted in distilled water to a final volume of one liter and then adjusted to pH 7.2) for 5–7 min at room temperature. This step was essential for the successful staining of the ganglionic cells bodies although immunoreactivity was seen in fibers in the nerve trunks without proteolosis. The tissues then were transferred from the protease solution directly into 4% paraformaldehyde in PBS at 4°C for 12 h. Following fixation, the ganglia were washed in four one hour baths of antisera diluent (ASD: 0.5% Triton X-100 and 1.0% normal goat serum in PBS) and then incubated for 72–84 h at 4°C in a 1:500 or 1:1000 dilution of primary antibody (rabbit anti-serotonin antibody from Immuno Nuclear Corp., Stillwater, Wisconsin) in ASD with 1.0% bovine serum albumin. No consistent difference in staining was noted between the different dilutions. The ganglia next were washed in four one-hour baths of ASD and then incubated for 24 h at 4°C in a 1:50 dilution of secondary antibody (goat anti-rabbit antibody conjugated to fluorescein isothiocyanate [FITC] from Antibodies, Inc. of Davis, California) in ASD. The ganglia were washed for 12 h in several changes of PBS and then cleared and mounted in 3:1 solution of glycerol in PBS.

The ganglia were viewed and photographed through a Leitz Orthoplan microscope equipped for epifluorescence (H2 filter block with an additional 455 nm longpass excitor filter). Photographs were made using Kodak Tri-X or Ektachrome 400 (daylight) film.

Controls for antibody selectivity

In addition to the ganglia from animals described above, several different groups of control ganglia were also processed. Except for the changes specifically mentioned below all other procedures for these control specimens were identical to those employed with the normal immunohistochemical preparations. In two groups, the primary anti-serotonin antibody was pre-incubated for 24 h at 4°C with 1 mg of serotonin creatinine sulfate or dopamine hydrochloride (Sigma) per ml of diluted antibody (1:500) prior to incubation of the ganglia. The final concentrations were thus 2.5×10^{-3} *M* serotonin and 5.2×10^{-3} *M* dopamine. Of these control ganglia the only ones to subsequently exhibit immunoreactivity were those ones of the group in which the primary antibody was pre-incubated with dopamine, and in this group the pattern of reactivity was similar to that observed following the normal procedure. No attempt was made to determine the minimum concentration of serotonin effective at blocking the staining, however, in a third control group all subsequent labelling was blocked when 1 ml of diluted primary antibody (1:1000) was pre-incubated with 400 μg serotonin conjugated to bovine serum albumin (Immuno Nuclear Corp.). In a fourth control group, incubation of the ganglia with the anti-serotonin antibody was replaced by an equal period of incubation with ASD alone. No fluorescent cell bodies or fibers were observed subsequently although a faint and diffuse fluorescent background was detected. A final control group was processed with the omission of the secondary antibody incubation. No significant fluorescence was subsequently observed in any of these ganglia either.

Results

Gross anatomy of the nervous system

The present findings on the anatomy of the nervous system of *Littorina* are in general agreement with previously published reports (Fretter and Graham, 1962; Bul-

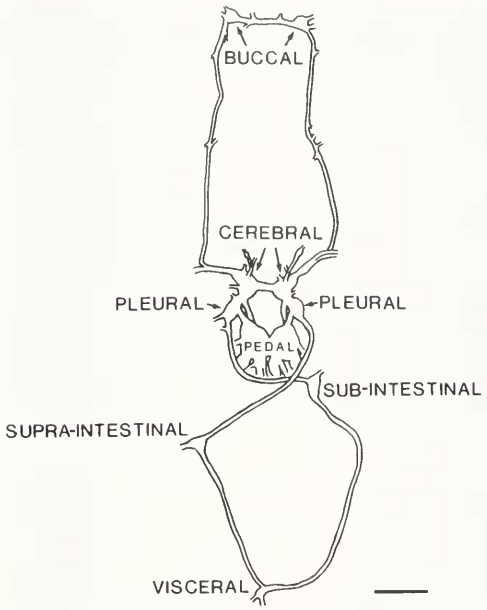


FIGURE 1. Dorsal view of the central nervous system of *Littorina*. Tracing was made from an enlargement of dissected tissue with the ganglia arranged to show approximate relative positions in the whole animal. The pedal ganglia, however, were rotated 90° so that the posterior side is facing upward. All ganglia in subsequent figures are shown in approximately the same orientation unless otherwise stated. Scale bar equals 1.5 mm.

lock and Horridge, 1965). In brief, the paired cerebral ganglia are located *in vivo* just posterior to the buccal mass and superior to the esophagous. These ganglia are connected to the paired buccal ganglia by two long cerebro-buccal connectives (CBC) which course through the superficial muscles of the buccal mass. Several small side branches from the CBC's innervate the buccal mass. Directly posterior to the cerebral ganglia are the two pleural ganglia which are connected to the cerebral ganglia by relatively short connectives. Unlike the other paired ganglia, the pleural ganglia are not interconnected by a commissure. Inferior and slightly posterior to the pleural ganglia are the pedal ganglia. The pedal ganglia are oriented with major anterior and posterior surfaces and with numerous roots exiting ventrally. They are connected to the cerebral and the pleural ganglia by the cerebro-pedal connectives (CPdC) and the pleuro-pedal connective (PlPdC), respectively. Apart from these paired ganglia, three smaller single ganglia are located more posteriorly. The subintestinal ganglion, which is found embedded in the muscle tissue of the right side of the foot, is connected to the left pleural ganglion by the pleuro-subintestinal connective (PlSbC). This connective crosses beneath the pleuro-supraintestinal connective (PlSpC) which connects the right pleural ganglion to the supraintestinal ganglion. The latter ganglion is located in the tissue underlying the left dorsal body wall. This resultant chiastoneury is characteristic of the mesogastropoda (Bullock and Horridge, 1965). The supraintestinal and the subintestinal ganglia are connected posteriorly to the visceral ganglion by the supraintestino-visceral connective (SpVC) and the subintestino-visceral connective (SbVC), respectively. A tracing of the entire central nervous system of *Littorina* is provided in Figure 1.

Localization of serotonin-like immunoreactivity

The distribution of serotonin-like immunoreactivity generally was similar, but not necessarily identical, in all animals. Many individual cells and clusters of cells could be identified reliably between specimens and these features are emphasized in the following descriptions. On the other hand, certain other cells and clusters of cells were noted only in some specimens. These variations are also noted. Using these techniques, a total of approximately 1500 cell bodies have been demonstrated to have serotonin-like immunoreactivity in the central nervous system of *Littorina*. While some neurites often could be traced from cell bodies to their terminations or to the nerve trunks by which they exited the ganglia, in most instances it was difficult to follow single neurites for long distances because they generally ran through neuropilar regions with numerous other immunoreactive elements. Furthermore, observations also were difficult in the cerebral ganglia due to the darkly pigmented cells in the overlying connective tissue.

Cerebral ganglia

Several clusters of cells were identified in the cerebral ganglia (Fig. 2a–e). One group of about 40 cells (the AM cluster) is located on the anterior medial margin of each ganglion (Fig. 2a–d). Cells of the AM cluster appeared to have processes which extend, in a discrete fascicle within the anterior portion of the cerebral commissure, to the contralateral ganglion. In addition to the commissural contributions, fibers also appeared to project from the AM cluster laterally along a prominent S-shaped tract toward the cerebro-buccal connective (CBC; Fig. 2a and 2d). It was not possible, however, to trace the neurite of any single AM cell directly into the CBC. In most instances, it was difficult to discern the outlines of many of the cell bodies in the AM cluster and usually little heterogeneity was seen. However, in certain specimens, two distinct sub-populations were apparent (Fig. 2b). One cluster of 3–5 larger cells lies more posteriorly, near the cerebral commissure, while another group of smaller, more anterior cells comprise the rest of the AM cluster.

Slightly posterior to the AM cluster, another group (the ventral medial or VM cluster) of about 30–40 cells was identified on the ventral surface of each cerebral ganglion. The somata of these cells are smaller than those of the AM group. The VM cluster appeared to have commissural fibers which form a bundle posterior to the afore-mentioned AM commissural fiberes. Some other fibers were traced to a neuropilar region at the lateral margin of the ganglion near a third cluster, the dorsal lateral (DL) group of cells. This DL cluster (Fig. 2b) has approximately 10–15 smaller cells which appeared to have neurites which project laterally toward the cerebro-pedal connective (CPdC).

Apart from these clusters, several prominent single cells also were identified. Among these are single, brightly labelled cells which easily can be identified along the posterior medial margin of each of the hemiganglia (Figs. 2a–c, e). While this pair of cells appeared to be quite consistent in size and shape between animals, exceptions were noted in which either the somatic shape or axonal projections differed. For instance, in Figure 2e, the initial segment of the neurite of one of the cells projects anteromedially rather than anterolaterally as is the case in cells shown in Figures 2a–c. Regardless of these differences, the initial neurite segment consistently branched at a point approximately 140 μm from the cell body. The medial branch then enters the cerebral commissure while the other branch enters the ipsilateral pleural ganglion via the CPdC (also see Fig. 5a). Lateral to these cells, two to four other large cells consis-

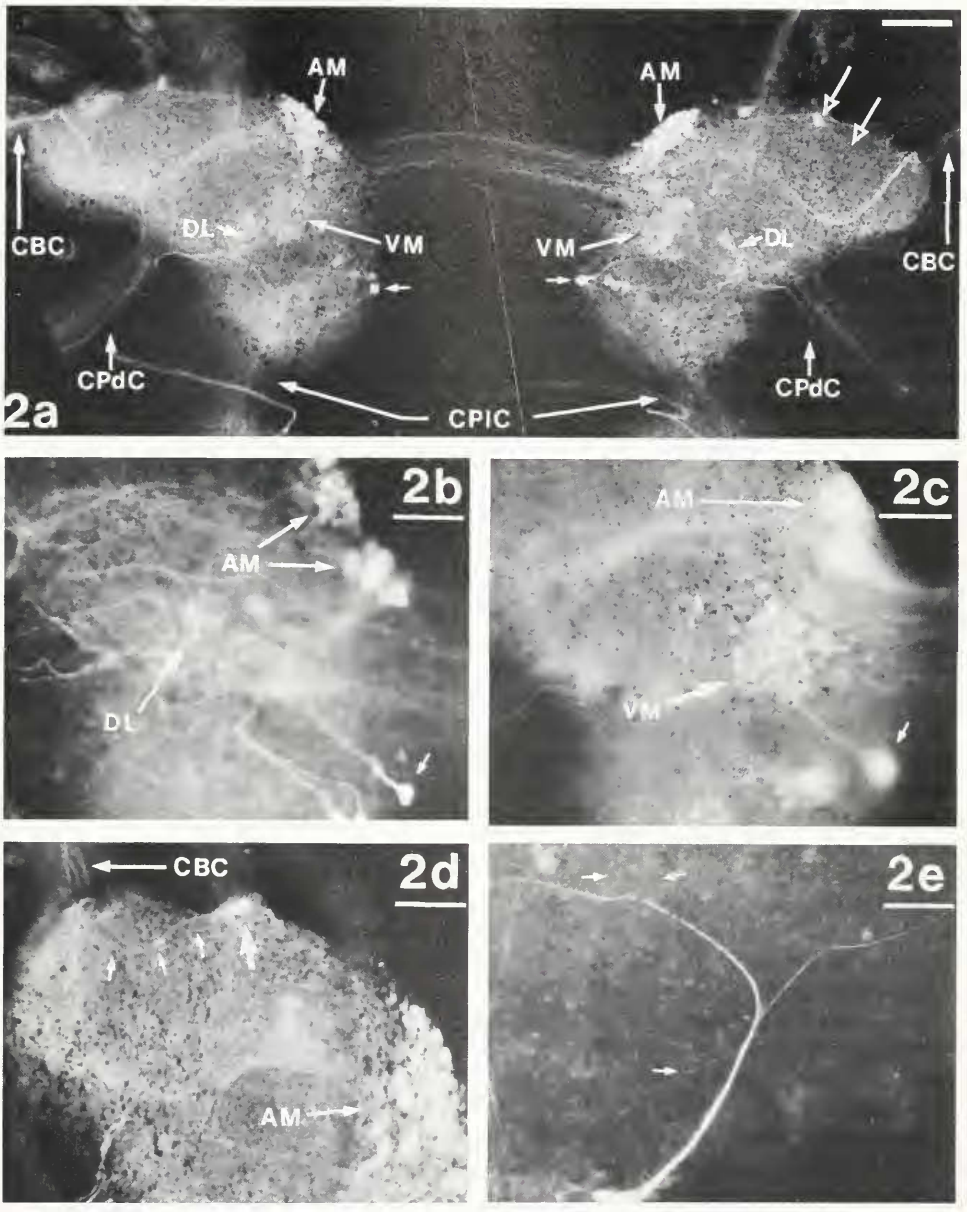


FIGURE 2. Serotonin immunoreactivity of cells in the cerebral ganglia. 2a: Several single cells and cell clusters can be differentiated. These include the anterior medial (*AM*), the ventral medial (*VM*), and the dorsal lateral (*DL*), clusters. Two particularly prominant, bilaterally symmetrical single cells along the posterior medial margin (small closed arrows) and another pair along the anterior margin (larger open arrows pointing to cell body and axon on right side) also can be seen. Interganglionic connectives labelled as follows: *CBC*, cerebrobuccal connective; *CPdC*, cerebropedal connective; *CPlC*, cerebropleural connective. Calibration bar equals approximately 130 μm. 2b–d: Higher magnifications of left cerebral ganglia from different individuals. Orientation is the same as in 2a. 2b and 2c show the DL and VM clusters in relation to the AM cluster and the prominant posterior medial cell (unlabelled arrows). 2d shows the cell body (large arrow) of the single anterior cell with a neurite (small arrows) which projects toward the CBC. Calibration bars for 2b–d = 75 μm. 2e: Details of the branching pattern of the posterior medial cell. Calibration bar for 2e = 45 μm.

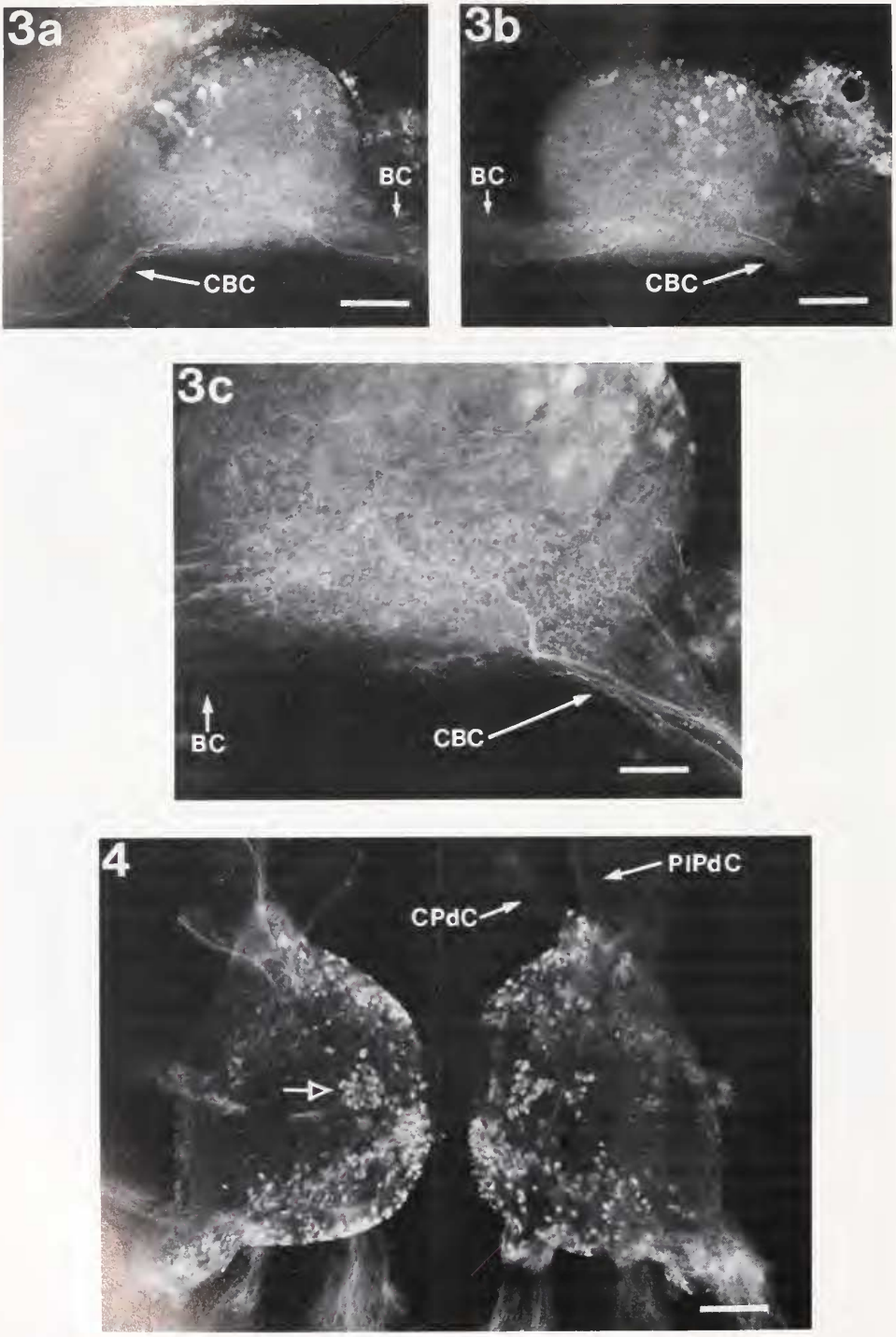


FIGURE 3. Immunoreactive cells in the left (3a) and right (3b) buccal ganglia. Scale bars equal 100 μm. Higher magnification (3c) shows details of fibers in the buccal ganglion. Scale bar = 44 μm. The buccal commissure, *BC*, and cerebrobuccal connective, *CBC*, are indicated.

tently were located in each cerebral ganglion. The processes of these cells appeared to extend toward the DL cluster as well as the posterior neuropilar region near the CPdC. A final pair of bilaterally symmetrical single cells also were found midway along the anterior margin of the ganglia between the AM cluster and the CBC. The cells were visible in approximately 90% of the specimens. Processes from each of these cells could be traced toward their ipsilateral CBC (Fig. 2a, d), however, despite numerous attempts, it was not possible to trace unmistakably the neurite of this cell directly into the CBC.

Buccal ganglia

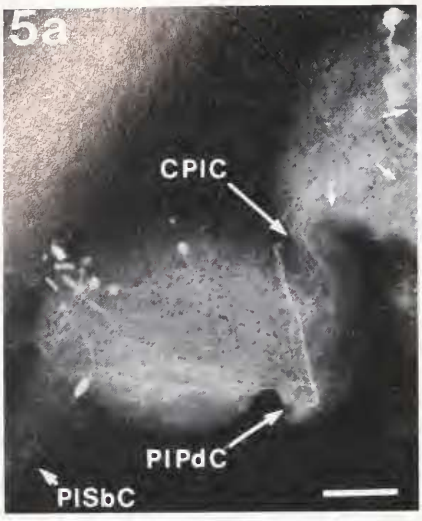
Several immunoreactive fibers can be discerned reliably along the lengths of each CBC (Figs. 2a, d and 3a–c). Once inside the buccal ganglia, these processes appeared to have some branches which terminate in the neuropile toward the posterior regions of the ganglia and also collaterals which transverse the buccal commissure toward the contralateral buccal ganglion (Figs. 3a–c).

A total of approximately 20–30 often faintly labelled cells were found in each of the paired buccal ganglia in approximately 75% of the specimens. In the remaining specimens, no cell bodies were discerned although numerous fibers stained prominently. When stained, the cell bodies were located mainly in the anterolateral quadrant of each of the ganglia (Figs. 3a–c). Processes from these cells appeared to project to the neuropile in the posterior part of the ganglia and possibly toward the buccal commissure (BC). Numerous immunoreactive fibers were seen reliably (not shown here) in many of the buccal roots, but it was not possible to determine their origins.

Pedal ganglia

The pedal ganglia have the largest number of immunoreactive cells of all the ganglia. A total of approximately 1000 cells were identified. The cell bodies are rather uniformly small with an average diameter of approximately 8 μm. While the cells lie in a fairly scattered distribution, some bilaterally symmetrical patterns of serotonin-like immunoreactivity could be discerned. One population of cells is distributed over the dorsal third of each pedal ganglion. It extends along the dorsomedial margin from the pedal commissure to the PlPdC. The band of cells then extends laterally along the anterior surface (and therefore is slightly out of focus in Fig. 4) from just dorsal to the pedal commissure to a point midway along the lateral margin of the ganglion. Processes from this population of cells were traced into both the PlPdC and the CPdC as well as a nerve trunk near the dorsolateral corner which innervates the periphery. A second population, forming a discrete cluster of 25–30 cells, is found on the posterior surface of the ganglion just lateral to the pedal commissure. Processes from this cluster were seen projecting principally towards the lateral margins of the ganglia and could be traced toward nerve trunks emerging from those regions. Further ventral, a third popoulation of cells was identified primarily along the posterior surface extending from the medial margin to the dorsolateral corner of the ganglia. This population appeared to be separable into an dorsal and a ventral band by a non-serotonergic region. The ventral band lies along the extreme ventral margin of the ganglion. While

FIGURE 4. Posterior view of the pedal ganglia. Dorsal is to the top of the figure. Open arrow indicates ventral cluster of cells in right pedal ganglion. The cerebropedal connective, *CPdC*, and pleuropedal connective, *PlPdC*, are indicated. Calibration bar = 160 μm.


5a
CPIC
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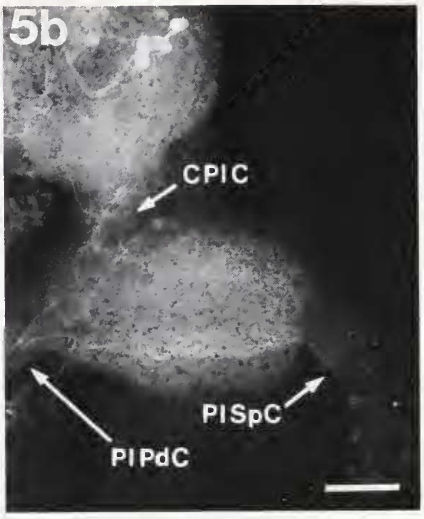
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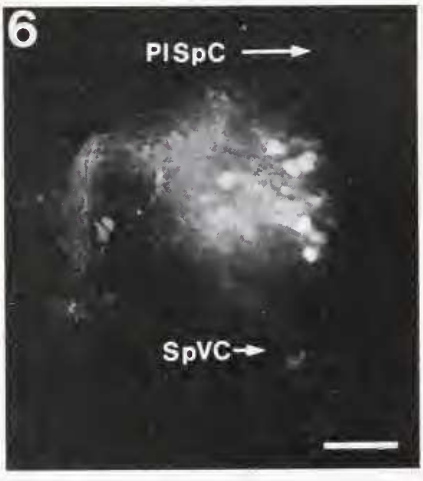
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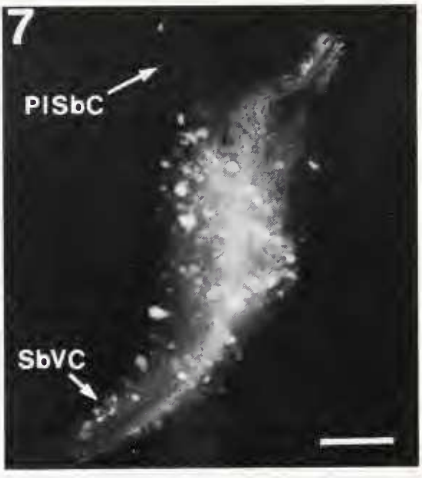
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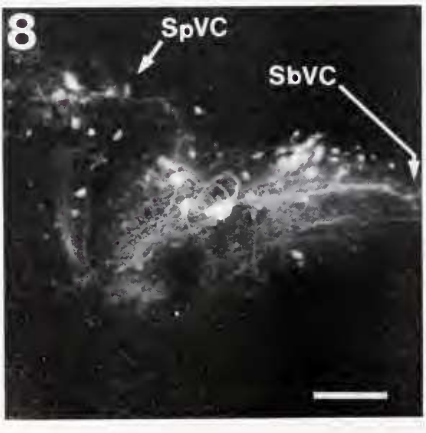
PISbC
SbVC


8
SpVC
SbVC

a few fibers from the ventral population were found in the ventral region of the pedal commissure, the majority of fibers were found in the nerve trunks emerging from the ventral margins of the ganglia.

Pleural ganglia

Although the two pleural ganglia are similar in size and shape, cell bodies reliably showing serotonin-like immunoreactivity were found principally in the left pleural ganglion. Approximately 10 labelled cells were reliable found in the anterolateral quadrant of this ganglion. Another few cells also were located occasionally along the anterior and the posterolateral margins. Processes from these cells appeared to project widely throughout the neuropile and also toward both the CPlC and the PlPdC (Fig. 5a). Although a few faintly labelled neurons occasionally were detected in the right pleural ganglia of some animals, in the majority of cases, these ganglia were devoid of immunoreactive cell bodies (Fig. 5b). However, labelled fibers reliably were found in all interganglionic connectives and intraganglionic fiber tracts associated with both the left and the right pleural ganglia.

Supraintestinal, subintestinal, and visceral ganglia

A cluster of approximately 40 cells are located along the medial border of the supra-intestinal ganglion (Fig. 6). Processes from this group were traced to the PlSpC and to a nerve trunk which exits from the lateral margin of the ganglion. A few cells were also found scattered lateral to this cluster. Processes from these cells were traced into the SpVC. Another group of 6–8 smaller cells were found near the anterior border of the ganglion. Processes from this group were found mainly in the nerve trunk on the lateral side of the ganglion.

Approximately 80 cells exhibiting serotonin-like immunoreactivity were located in the subintestinal ganglion (Fig. 7). Bundles of fibers were seen in both the PlSbC and the SbVC. Within the ganglion, these fibers appeared to be confined to the neuropile at the center of the ganglion. It was impossible to determine the origins of these fibers.

Numerous immunoreactive cells were found in the visceral ganglion (Fig. 8). In the center of the ganglion, a few cells with relatively large somata were prominent. These cells appeared to have processes extending toward the SpVC, the SbVC, and a nerve trunk exiting the ganglion at the posterior margin of the ganglion. Laterally, a cluster of approximately 30 cells was observed on the right side of the ganglion where the SbVC joins the ganglion. Another population of approximately 10–20 cells was located on the left anterolateral margin where the SpVC joins the visceral gan-

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FIGURE 5. Immunoreactivity in the left (5a) and right (5b) pleural ganglia. Also indicated (small arrows in 5a) is lateral branch of the neurite of prominant posterior medial cell of the cerebral ganglion which projects to the pleural ganglion. Interganglionic connectives are labelled as follows: *CPlC,* cerebropleural connective; *PlPdC,* pleuropedal connective; *PlSbC,* pleurosubintestinal connective; *PlSpC,* pleurosupraintestinal connective. Scale bar = 100 μm.

FIGURE 6. Serotonin immunoreactive cells in the supraintestinal ganglion. The pleurosupraintestinal connective, *PlSpC,* and the supraintestinovisceral connective, *SpVC,* are shown. Scale bar = 100 μm.

FIGURE 7. Immunoreactivity in the subintestinal ganglion. The pleurosubintestinal connective, *PlSbC,* and the subintestinovisceral connective, *SbVC,* are indicated. Calibration bar = 100 μm.

FIGURE 8. Serotonin immunoreactivity in the visceral ganglion. The supraintestinovisceral and the subintestinovisceral connectives, *SpVC* and *SbVC,* respectively, are indicated. Scale bar = 100 μm.

glion. Posteriorly, a few fibers were traced into a nerve trunk which innervates the periphery.

Discussion

Recently developed immunocytochemical techniques provide a simple, reliable method to rapidly compare the nervous systems of diverse species at the level of the single identified cells or clusters of small numbers of cells. Relatively small numbers of cells with distinct biochemical phenotypes can be selectively revealed. When applied to whole-mounted ganglia, these techniques allow for easy visualization of the three dimensional structure of cells in preparations with a close correspondence to the preparations used in physiological studies. Thus they reveal morphology and biochemistry and allow for a correlation with function. The identification of such biochemically discrete single cells or small populations of cells with known functions carries added importance in that it allows for comparisons of specific, analogous, and possibly homologous, cells across phylogeny.

The use of this approach to tentatively identify the serotonergic cells of the gastropod nervous system appears to be particularly promising. Serotonin has long been thought to play an important role in the gastropod nervous system (Gerschenfeld, 1973). More recently, several serotonergic cells or clusters of cells have been identified in diverse species and it has been possible to ascribe a role to many of these cells. For example, activity in the serotonergic metacerebral giant cells modulates the patterned motor output which underlies feeding in numerous gastropod species including *Aplysia* (Weiss *et al.,* 1978, 1979), *Pleurobranchaea* (Gillette and Davis, 1977; Croll *et al.,* 1985), Limax (Gelperin, 1981), and *Helisoma* (Granzow and Kater, 1979). Identified serotonin-containing cells in the pedal ganglia of *Tritonia* initiate locomotion by activating ciliary cells on the sole of the foot (Audesirk, 1978; Audesirk *et al.,* 1979). Evidence suggests that other serotonergic cells function as cardio-excitors (Liebeswar *et al.,* 1975) and others may play a role in re-setting circadian rhythms (Corrent *et al.,* 1978).

Immunohistochemical techniques allow for the initial identification of serotonergic cells and thus serve as a basis for direct comparison of the nervous systems of phyletically distant species such as *Littorina.* However, observations of serotonin-like immunoreactivity must be interpreted in the light of demonstrated selectivity of the procedure. Several lines of evidence indicate that the immunocytochemical techniques employed in this study appear to reliably detect serotonergic cells. The controls within this study demonstrate that selective binding of the primary antibody to tissue antigens underlies this procedure and that this binding of the primary antibody, at the dilutions employed here, can be blocked by pre-incubation with serotonin conjugated to bovine serum albumin or with free serotonin but not with dopamine. This finding is consistent with results found in other invertebrate studies (Beltz and Kravitz, 1983; Goldstein *et al.,* 1984; Klemm *et al.,* 1984; Ono and McCaman, 1984). More direct evidence for the specificity of the immunohistological technique for the demonstration of serotonin in invertebrate nervous systems comes from the close correlation found between the distribution of serotonin-like immunoreactivity in *Aplysia* and the serotonin contents of single identified cells as determined by a radioenzymatic assay (Ono and McCamman, 1984). There have also been good correlations between cells which show serotonin-like immunoreactivity and serotonin-like (yellow) fluorescence obtained through glyoxylic acid and aldehyde demonstrations of biogenic amines in *Aplysia* (Tritt *et al.,* 1983; Ono and McCaman, 1984) and in the cockroach (Klemm, 1983; Klemm *et al.,* 1984). In both animals, however,

immunohistochemistry labelled more cells, thereby indicating either its greater sensitivity or lesser selectivity. The former possibility seems more likely given the other available evidence.

Assuming, then, that serotonin-like immunoreactivity is a good indicator of serotonin content, one can make meaningful comparisons in staining patterns between species. A notable feature of the central ganglia in *Littorina* is that many more cells appear to be serotonergic than in representative opisthobranch and pulmonate species. For example, Ono and McCaman (1984) estimated a total of only approximately 120 serotonergic central neurons in *Aplysia* based on their immunohistological studies. Audesirk (1985) appears to have located approximately 200 serotonergic cells in *Lymnaea* based on glyoxylic acid histofluorescence. By contrast, our evidence suggests that the central ganglia of *Littorina* contain a total of approximately 1500 serotonergic cells. While a portion of this total number is composed of cells which exhibited variable labelling intensity and therefore may contain questionable levels of serotonin (see below), the vast majority (over 90%) reliably exhibited intense and unambiguous staining. Therefore it appears that the nervous system of this prosobranch contains 7–10 times as many serotonergic cells as do the nervous systems of representative pulmonates and opisthobranchs.

Another general feature of the nervous system of *Littorina,* which is a well known feature of prosobranchs (Bullock and Horridge, 1965) and which was confirmed here is that the neuronal cell bodies are relatively small as compared to the giant cell bodies found in the nervous systems of the pulmonates and opisthobranchs. While much of the difference in cell size may be accounted for by the small body size of *Littorina,* a comparison of the relative sizes of the serotonergic cell bodies to ganglion dimensions in adult *Aplysia* (Ono and McCaman, 1984), juvenile *Aplysia* (Goldstein *et al.,* 1984), and adult *Lymnaea* (Audesirk, 1985) all emphasize the predominance of relatively smaller serotonergic cells in the nervous system of *Littorina.*

The larger number and the smaller relative sizes of the serotonergic cells indicate that cell-to-cell correspondences may not always exist, or may be difficult to recognize between *Littorina* and any pulmonate or opisthobranch species. However, more general comparisons can be made.

Several clusters of cells and a few identified single cells have been located in the cerebral ganglia of *Littorina.* As in the other gastropod species thus far examined, the cerebral ganglia appear to be bilaterally symmetrical in their serotonergic innervation. Projections from the cerebral ganglia are generally poorly described in other species, however, one well-known serotonergic pathway from the cerebral ganglia involves the metacerebral giant cell (MCG). In other species, the MCG appears to be the only source of serotonin in the buccal ganglion (Ono and McCaman, 1984; Goldstein *et al.,* 1984, Murphy *et al.,* 1985). Fibers from the cerebral ganglia appear to be a major but perhaps not the only source of serotonergic innervation to the buccal ganglia in *Littorina.* However, unlike the case in other species where the only serotonergic fiber in the each of the CBC's is the single, large axon of the MCG, in *Littorina* each of the CBC's contains several fibers with serotonin-like immunoreactivity. The source(s) of these fibers is still unclear but they may arise from one or more of the following three sources: (1) a single, isolated cell body lying midway along the anterolateral margin of the cerebral ganglion, (2) cells within the AM cluster of the cerebral ganglion, or (3) a population of cells residing within the buccal ganglia. With regard to this last cell population exhibiting serotonin-like immunoreactivity, the buccal ganglia of *Littorina* appear to differ from the buccal ganglia of the other species thus far examined, where no evidence exists for serotonergic cell bodies in normal animals. Interestingly, however, Audesirk (1985) found that when the buccal ganglia

of *Lymnaea* were pre-incubated in 5-hydroxytryptophan before histological processing, a small number of cells subsequently showed serotonin-like fluorescence induced by glyoxylic acid. No serotonergic cell bodies were detected in the buccal ganglia if the pre-incubation was omitted. This evidence suggests that certain cells in the buccal ganglia of *Lymnaea* differ in their uptake of this serotonin precursor. It is unknown whether these cells subsequently synthesized serotonin since glyoxylic acid induced histofluorescence does not allow for discrimination between 5-hydroxytryptophan and serotonin.

While serotonin-like immunoreactivity appears to correspond generally well with serotonin content, it must be noted that cells in the buccal ganglia were variable in their staining intensity. They were often labelled only faintly and often were unlabelled. Despite this variability, the immunoreactive fibers in the buccal roots and neuropile showed relatively little variability. Ono and McCaman (1984) reported a certain degree of variability in both serotonin-like immunoreactivity and in serotonin content of individual neurons throughout the central ganglia of *Aplysia*. It is unknown whether physiological changes in precursor availability may affect the distribution of serotonin-like immunoflourescence in gastropods but these data suggest a possible correspondence between the buccal cells in *Littorina* which show variable serotonin-like immunoreactivity and buccal cells in *Lymnaea* which can express selective serotonin precursor uptake and/or serotonin synthesis capabilities.

In other species the pedal ganglia appear to contain the largest number of serotonergic neurons. Certain of these cells may be involved in ciliary locomotion in both *Tritonia* (Audesirk, 1978; Audesirk *et al.*, 1979) and *Lymnaea* (Audesirk, 1985). There is also some evidence that serotonin may be involved in controlling the locomotory activity emerging from the pedal ganglia of *Aplysia* (MacKey and Carew, 1983). In *Littorina* the pedal ganglia are also the major source of innervation of the foot (Fretter and Graham, 1962; Bullock and Horridge, 1965) and the pedal ganglia contain the largest number of cells with serotonin-like immunoreactivity. No physiological role for serotonin in *Littorina* is known at present.

The pleural ganglia seem to have very few serotonergic cell bodies, in other gastropod species. Relatively few serotonergic cells were found in the pleural ganglia of *Littorina* as well. However, a few cells were reliably labelled. Interestingly, among the paired anterior ganglia, which all superficially appear to be bilaterally symmetrical, only the pleural ganglia are asymmetrical in serotonin-like immunoreactivity distribution. This asymmetry is presumably a reflection of the fact that the more posterior ganglia which show very little bilateral symmetry are connected directly to the pleural ganglia. These posterior ganglia, the supraintestinal, subintestinal, and visceral ganglia, each have numerous elements exhibiting serotonin-like immunoreactivity. Similarly serotonin appears to be widely distributed in the more posterior ganglia innervating the viscera of other gastropods (Goldstein *et al.*, 1984; Ono and McCaman, 1984; Audesirk, 1985). Unfortunately, too little is presently known about the innervation patterns of the viscera in *Littorina* to make any meaningful comparisons with other species.

The present paper demonstrates that immunocytochemical techniques for the localization of serotonin can provide a basis for comparisons of phyletically distant species. The technique reveals general serotonin distribution patterns within central ganglia and in some cases can allow for the identification of individual cells. Details of neurite projections and arborizations also can be visualized. Further information on the physiological function of these neuronal elements are needed. Further studies on other prosobranch nervous systems also are needed in order to test the generality of the findings reported here. Work presently in progress is focused towards this goal.

It is expected that such studies on the localization of discrete neurochemical substances, such as serotonin, and accompanying physiological studies conducted in a broader phyletic survey will yield new information on the evolutionary trends which have shaped the gastropod nervous system.

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